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(54) Title: A DETERGENT CONTAINING A PROTEASE AND A PROTEASE INHIBITOR AND NOVEL INHIBITORS FOR USE THEREIN

(57) Abstract

Detergent composition comprising a protease and a modified subtilisin inhibitor of Family VI having Pro as the P1 residue in combination with one or more of the following amino acid substitutions as the indicated positions: P4: Pro; P3: Tyr, Glu, Ala, Arg or Pro; P2: Arg, Pro, Glu, Val or Tyr.

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A DETERGENT CONTAINING A PROTEASE AND A PROTEASE INHIBITOR AND NOVEL INHIBITORS FOR USE THEREIN

TECHNICAL FIELD

This invention relates to an improved detergent composition comprising a protease (particularly a subtilisin) and a reversible protease inhibitor of peptide or protein type, to a detergent additive comprising such a protease and inhibitor and to a method for stabilizing a protease.

The invention also relates to novel modified subtilisin inhibitors for use in said detergent, to a recombinant DNA molecule comprising a nucleotide sequence coding for the modified subtilisin inhibitor, to a transformed host organism comprising the DNA and to a method of producing the modified inhibitor.

BACKGROUND ART

Proteases, especially subtilisins, are widely used as ingredients in commercial detergents. A major problem in formulating protease-containing detergents, especially liquid detergents, is that of ensuring enzyme stability during storage.

The prior art has dealt extensively with improving the storage stability of enzymes. As an example, JP-A 62-269689 demonstrates improvement of the stability of a protease (e.g. a subtilisin) in a liquid detergent by incorporation of a protease inhibitor of protein type. As stated in said publication, the protease inhibitor should ideally show essentially no inhibiting effect under dilute washing conditions, i.e. when the detergent is in use.

STATEMENT OF THE INVENTION

We have found that in the known detergents containing protease and inhibitor, the protease is almost totally inhibited under dilute washing conditions. We have also found that by a suitable choice of inhibitor for a given protease, it is possible to essentially avoid inhibition at the dilute conditions of washing, while still achieving effective enzyme stabilization in the detergent during storage.

We have also found that subtilisin inhibitors with this improved performance can be derived from known inhibitors by substituting certain amino acids. The novel inhibitors can be produced by known protein engineering methods.

Accordingly, the invention provides a detergent composition comprising a protease and a modified subtilisin inhibitor of Family VI having Pro as the P1 residue in combination with one or more of the following amino acid substitutions at the indicated positions:

P4: Pro,
P3: Tyr, Glu, Ala, Arg, Pro,
P2: Arg, Pro, Glu, Val, Tyr,

Using the novel inhibitor, the ratio of the dissociation constant to the protease concentration will be in the range from 0.006 to 6, or the dissociation constant will be in the range from 1 nM to 50 μM. The invention also provides a detergent additive comprising protease in the form of a stabilized liquid or a non-dusting granulate, further comprising the modified subtilisin inhibitor.

The invention also provides a modified subtilisin inhibitor of family VI, as defined above, excluding:

Eglin B and C substituted with
Pro at position 44 (P2).

Further, the invention provides a recombinant DNA molecule comprising a nucleotide sequence coding for a modified subtilisin inhibitor as defined above, a transformed host organism comprising said DNA and a method of producing the modified inhibitor comprising cultivation of the transformed host organism.

Modified subtilisin inhibitors of family VI are known (EP 332,576, C. Langstaff et al., *Biochemistry*, **1990**, *29*, 7339-7347), but their use in detergents and the resulting advantages have not been disclosed or suggested.

DETAILED DESCRIPTION OF THE INVENTION

Protease

The protease used in the invention is preferably of microbial origin. It may be a serine protease, preferably an alkaline microbial protease or a trypsin-like protease. Examples of alkaline proteases are subtilisins, especially those derived from *Bacillus*, e.g. subtilisin Novo, subtilisin Carlsberg, subtilisin 309, subtilisin 147 and subtilisin 168 (both described in WO 89/06279) and mutant subtilisins such as those described in WO 89/06279 and DK 0541/90. Examples of commercial *Bacillus* subtilisins are Alcalase®, Savinase® and Esperase®, products of Novo Nordisk A/S. Examples of trypsin-like proteases are trypsin (e.g. of porcine or bovine origin) and the *Fusarium* protease described in WO 89/06270.

The amount of protease in the detergent will typically be 0.2-40 μM, especially 1-20 μM (generally 5-1000 mg/l, especially 20-500 mg/l) as pure enzyme protein.

Inhibitor

According to the invention, the inhibitor is chosen for a given detergent (protease type and concentration etc.) so that the dissociation constant (K_d) is high enough to allow adequate release of protease when the detergent is diluted with water, yet the dissociation constant is low enough to allow efficient

inhibition in the concentrated detergent during storage. K_d is commonly defined for a given protease and a given inhibitor in a given system as the equilibrium constant

$$K_d = [E] * [I] / [EI]$$

where the square brackets indicate molar concentration of free enzyme (E), free inhibitor (I) and enzyme-inhibitor complex (EI), respectively.

The ratio of the dissociation constant to the protease concentration is preferably from 0.06 to 6. The dissociation constant is preferably from 1 to 10 μM (i.e. 10^{-6}-10^{-5} M).

The amount of inhibitor is preferably such that the molar ratio of inhibitor reactive site to protease active site is above 0.6, preferably 1-10.

Novel inhibitor

The novel inhibitors provided by the invention may be derived from the known inhibitors of Family VI, described in the above-mentioned references, e.g. from barley subtilisin inhibitor CI-1 or CI-2, potato subtilisin inhibitor (PSI), Eglin B or C, tomato subtilisin inhibitor or *Vicia* subtilisin inhibitor (VSI).

Inhibitors of this family are known to strongly inhibit the subtilisins commonly used in detergents, with inhibitor dissociation constants generally below 10^{-10} M. We have found that by using these inhibitors to stabilize a protease in a detergent, the protease is so strongly bound that very little protease activity is released when the detergent is diluted for use in washing, and the protease remains almost completely inactive. We have therefore realized a need for a modified inhibitor with weaker binding to the protease.

We have previously found that the protease-inhibitor binding can be suitably weakened by substituting the P1 residue with Pro (starting from the reactive site, amino acids positions are numbered P1, P2 etc. in the direction of the N-terminal). This modified inhibitor is resistant to hydrolysis by the protease. We have now found that the protease-inhibitor binding can be further weakened by a combination of Pro as the P1 residue and one or more of the following amino acid substitutions at the indicated positions.

P4: Pro,

P3: Tyr, Glu, Ala, Arg, or Pro,

P2: Arg, Pro, Glu, Val, or Tyr

The novel inhibitors may be produced by known recombinant DNA techniques. Briefly, a DNA sequence (cDNA or a synthetic gene) encoding a known inhibitor is subjected to mutagenesis in order to replace the codon(s) for the amino acid(s) to be substituted with a new codon (codons) for the desired amino acid substitution(s). This may preferably be carried out by oligonucleotide-directed site-specific mutagenesis in bacteriophage M13 vectors (e.g. M.J. Zoller and M. Smith, Meth. Enzymol. 100 (1983) 468-500), in double-stranded DNA vectors (e.g. Y. Morinaga et al., Biotechnology (July 1984) 636-639), or by the polymerase chain reaction (PCR) (e.g. R. Higuchi, Nucl. Acids. Res. 16 (1988) 7351-7367).

The mutant gene is subsequently expressed in a suitable host strain. Suitable hosts are bacteria (e.g. strains of *Escherichia coli* or *Bacillus*), fungi (e.g. strains of *Saccharomyces cerevisiae* or filamentous fungi like *Aspergillus*), plants such as tomato or potato or established human or animal cell lines. To accomplish expression, the mutant gene has to be inserted in an expression plasmid with promoter and terminator DNA elements for the formation of translatable mutant inhibitor mRNA *in vivo*. The plasmid is introduced into the host by genetic transformation. The choice of expression plasmid is dependent on the type of host strain used. The expression of the mutant inhibitor may be done intracellularly or extracellularly. In the latter case, the DNA sequence coding for the mutant inhibitor is fused in frame to a DNA sequence encoding a suitable peptide signalling secretion. The secretion signal should preferably be cleaved off *in vivo*, resulting in secretion of the mature mutant inhibitor into the growth medium.

Various species of Bacilli, including *Bacillus alkalophilus*, *B. amyloliquefaciens*, *B. brevis*, *B. lentus*, *B. licheniformis*, *B. megaterium*, *B. stearothermophilus*, and *B. subtilis*, are known to secrete proteins efficiently. In many cases this has also been shown to be the case for heterologous proteins.

Since expression of a secreted protease inhibitor has the potential advantage of facilitating purification, it is obviously interesting to attempt to express the inhibitor as a secreted product from a *Bacillus* strain. This could for instance be accomplished by combining the structural part of the inhibitor with the promoter and signal peptide of a well expressed and secreted *Bacillus* enzyme as for instance the maltogenic amylase from *B. stearothermophilus* (Diderichsen, B. and Christiansen, L. Cloning of a maltogenic α-amylase from *Bacillus stearothermophilus*, FEMS Microbiol. Lett. 56:53-60. 1988) or the α-amylase from *B. licheniformis* (Jørgensen, P.L., C.K. Hansen, G.B. Poulsen and B. Diderichsen. *In vivo* genetic engineering: Homologous recombination as a tool for plasmid construction, GENE 96: 37-41, 1990). This may be accomplished in many ways as known by persons skilled in the art. One way is to use *in vivo* genetic engineering (Jørgensen et al. 1990, op. cit.). The advantage of this method is that it easily generates a perfect fusion between signal peptide and mature inhibitor which according to well documented rules for signal peptide processing would be expected to give the correct N-terminal amino acid residue of the inhibitor.

In one method of producing variants of barley CI-2A inhibitor a filamentous fungus is used as the host organism. The filamentous fungus host organism may conveniently be one which has previously been used as a host for producing recombinant proteins, e.g. a strain of *Aspergillus sp.*, such as *A. niger*, *A. nidulans* or *A. oryzae*. The use of *A. oryzae* in the production of recombinant proteins is extensively described in, e.g. EP 238 023.

For expression of CI-2A inhibitor variants in *Aspergillus*, the DNA sequence encoding the protease inhibitor is preceeded by a promoter. The promoter may be any DNA sequence exhibiting a strong transcriptional activity in Aspergillus and may be derived from a gene encoding an extracellular or intracellular protein such as an amylase, a glucoamylase, a protease, a lipase, a cellulase or a glycolytic enzyme.

Examples of suitable promoters are those derived from the gene encoding *A. oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *A. niger* neutral α-amylase, *A. niger* acid stable α-amylase, *A. niger* glucoamylase,

Rhizomucor miehei lipase, *A. oryzae* alkaline protease or *A. oryzae* triose phosphate isomerase.

In particular when the host organism is *A. oryzae*, a preferred promoter for use in the process of the present invention is the *A. oryzae* TAKA amylase promoter as it exhibits a strong transcriptional activity in *A. oryzae*. The sequence of the TAKA amylase promoter appears from EP 238 023.

Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

To ensure secretion of the inhibitor or variants hereof from the host cell, the DNA sequence encoding the inhibitor may be preceded by a signal sequence which may be a naturally occurring signal sequence or a functional part thereof or a synthetic sequence providing secretion of the protein from the cell. In particular, the signal sequence may be derived from a gene encoding an *Aspergillus sp.* amylase or glucoamylase, a gene encoding a *Rhizomucor miehei* lipase or proteinase, or a gene encoding a *Humicola* cellulase, xylanase or lipase.

Detergent

The detergent of the invention may be in any convenient form, e.g. as powder, granules or liquid. A liquid detergent may be aqueous, typically containing up to 70% water and 0-20% organic solvent.

The detergent comprises a surfactant which may be anionic, non-ionic, cationic, amphoteric or a mixture of these types. The detergent will usually contain 5-30% anionic surfactant such as linear alkyl benzene sulphonate (LAS), alpha-olefin sulphonate (AOS), alkyl sulphate (AS), alcohol ethoxy sulphate (AES) or soap. It may also contain 3-20% non-ionic surfactant such as nonyl phenol ethoxylate or alcohol ethoxylate.

The detergent composition may additionally comprise one or more other enzymes, such as an amylase, lipase, cellulase or peroxidase.

The pH (measured in aqueous detergent solution) will usually be neutral or alkaline, e.g. 7-10. The detergent may contain 1-40% of a detergent builder such as zeolite, phosphate, phosphonate, citrate, NTA, EDTA or DTPA, alkenyl succinic anhydride or silicate, or it may be unbuilt (i.e. essentially free of a

detergent builder). It may also contain other conventional detergent ingredients, e.g. bleaching agents or bleach precursors or a system comprising a bleaching agent and/or precursor together with an activator therefor, fabric conditioners, foam boosters, anti-corrosion agents, soil-suspending agents, sequestering agents, anti-soil redeposition agents, stabilizing agents for the enzyme(s), foam depressors, dyes, bactericides, optical brighteners or perfumes.

Particular forms of detergent composition within the scope of the invention include:

a) A detergent composition formulated as a detergent powder containing phosphate builder, anionic surfactant, nonionic surfactant, acrylic or equivalent polymer, perborate bleach precursor, amino-containing bleach activator, silicate or other structurant, alkali to adjust to desired pH in use, and neutral inorganic salt.

b) A detergent composition formulated as a detergent powder containing zeolite builder, anionic surfactant, nonionic surfactant, acrylic or equivalent polymer, perborate bleach precursor, amino-containing bleach activator, silicate or other structurant, alkali to adjust to desired pH in use, and neutral inorganic salt.

c) A detergent composition formulated as an aqueous detergent liquid comprising anionic surfactant, nonionic surfactant, humectant, organic acid, caustic alkali, with a pH adjusted to a value between 9 and 10.

d) A detergent composition formulated as a nonaqueous detergent liquid comprising a liquid nonionic surfactant consisting essentially of linear alkoxylated primary alcohol, triacetin, sodium triphosphate, caustic alkali, perborate monohydrate bleach precursor, and tertiary amine bleach activator, with a pH adjusted to a value between about 9 and 10.

e) A detergent composition formulated as a detergent powder in the form of a granulate having a bulk density of at least 600 g/l, containing anionic surfactant

and a mixture of nonionic surfactants with respective alkoxylation degrees about 7 and about 3, low or substantially zero neutral inorganic salt, phosphate builder, perborate bleach precursor, tertiary amine bleach activator, sodium silicate, and minors and moisture.

f) A detergent composition formulated as a detergent powder in the form of a granulate having a bulk density of at least 600 g/l, containing anionic surfactant and a mixture of nonionic surfactants with respective alkoxylation degrees about 7 and about 3, low or substantially zero neutral inorganic salt, zeolite builder, perborate bleach precursor, tertiary amine bleach activator, sodium silicate, and minors and moisture.

g) A detergent composition formulated as a detergent powder containing anionic surfactant, nonionic surfactant, acrylic polymer, fatty acid soap, sodium carbonate, sodium sulphate, clay particles, perborate bleach precursor, tertiary amine bleach activator, sodium silicate, and minors and moisture.

h) A detergent composition formulated as a detergent (soap) bar containing soap based on pan-saponified mixture of tallow and coconut oil, neutralised with orthophosphoric acid, mixed with protease, also mixed with sodium formate, borax, propylene glycol and sodium sulphate, and then plodded on a soap production line.

Apart from these ingredients, the detergent compositions a)-h) include a protease and a modified subtilisin inhibitor of Family VI as described above, and optionally one or more other enzymes, as indicated above.

Other examples of detergents according to the invention may be obtained from the compositions disclosed in WO 89/04361, DK 5111/89 or PCT/DK91/00243 by incorporating protease and inhibitor according to the invention. PCT/DK91/00243 is incorporated herein by reference.

The invention is particularly applicable to the formulation of detergents with pronounced enzyme stability problems, e.g those containing oxidizing

agents. Such det rgents typically contain 1-40%, especially 5-20% oxidizing agent. They may be granular detergents containing granules of a perborate or percarbonate and separate granules containing enzyme and inhibitor according to the invention, or they may be aqueous or non-aqueous liquid detergents containing hydrogen peroxide, a perborate or a percarbonate (see e.g. EP 378,261, EP 378,262, EP 294,904, EP 368,575).

Detergent additive

The protease and inhibitor may be included in the detergent of the invention by separate addition or by adding the combined additive provided by the invention. The additive will usually contain 0.2-8 mM protease (0.5-20%) and have an inhibitor/protease ratio as described above.

The detergent additive may be in liquid form for incorporation in a liquid detergent. A liquid additive may contain 20-90% propylene glycol; 0.5-3% (as Ca) of a soluble calcium salt; 0-10% glycerol; minor amounts of short-chain fatty acids and carbohydrate; and water up to 100%.

EXAMPLE 1

Expression of barley subtilisin inhibitor CI-2A in *Saccharomyces cerevisiae*

The barley subtilisin inhibitor and variants hereof according to the invention can be produced biosynthetically in a yeast host expressing a DNA sequence encoding the inhibitor.

To achieve secretion to the growth medium, the DNA sequence encoding the inhibitor can be fused to another DNA-sequence encoding a signal peptide functional in yeast. An example hereof is the *Saccharomyces cerevisiae* MFα-1 leader sequence (Kurjan & Herskowitz, Cell 30, 933-943 (1982). A preferred construction uses the DNA sequence encoding the entire 85 aminoacid MFα-1 leader sequence including the dibasic site LysArg. In that way, efficient secretion of CI-2A inhibitor with the correct N-terminal is achieved.

Plasmid construction

All expression plasmids are of the C-POT type. Such plasmids are described in EP patent application No. 85303702.6 and are characterized in containing the *S. pombe* triose phosphate isomerase gene (POT) for the purpose of plasmid stabilization. A plasmid containing the POT-gene is available from a deposited *E.coli* strain (ATCC 39685). The plasmids furthermore contain the *S. cerevisiae* triose phosphate isomerase promoter and terminater (P_{TPI} and T_{TPI}). They are identical to pLaC200 described in the patent application WO 89/02463, except for the region defined by the EcoRI/XbaI restriction fragment encoding a signal/leader/insulin precursor sequence. In this application, the region is replaced by a fragment encoding the MFα-1 leader fused to the inhibitor sequence. The sequence of the fragment is shown in Sequence Listing ID No. 1 (P1 is located at Met 59). The isolation of the barley CI-2A subtilisin inhibitor cDNA is described by Williamson et al. Eur. J. Biochem. 165, 99-106 (1987). Cloning of the MFα-1 leader is described by Kurjan & Herskowitz (reference given above). Modifications and assembly of the two sequences were carried out using entirely standard techniques. In particular, the KpnI and ClaI restriction sites at positions 495 and 519, respectively, were generated by introducing silent mutations into the inhibitor gene. This was done by *in vitro* mutagenesis of the inhibitor gene. A map of the expression plasmid pYACI2 is shown in Figure 1.

Introduction of mutations into the inhibitor gene

Mutant CI-2A genes were generated using PCR mutagenesis, which was carried out as follows: A primer carrying the mutation flanked by homologous sequences and carrying the introduced KpnI-site was used together with another primer homologous to sequences in the T_{TPI} region in a PCR amplification reaction. In that way, fragments were generated which contained the desired mutations. The ends were trimmed with the restriction enzymes KpnI and XbaI, purified on agarose gels, and cloned into pYACI2 previously digested with the same restriction enzymes. The presence of the mutation was verified by DNA sequencing. The primers used are listed below.

Mutation	Primer sequence
P1Pro,P4Pro	5'-CCGGTGGGTACCCCAGTGACCCCAGAATATCGGATC-3'
P1Pro,P3Tyr	5'-CCGGTGGGTACCATTTACACCCCAGAATATCGGATC-3'
P1Pro,P3Glu	5'-CCGGTGGGTACCATTGAAACCCCAGAATATCGGATC-3'
P1Pro,P3Arg	5'-CCGGTGGGTACCATTAGAACCCCAGAATATCGGATC-3'
P1Pro,P3Ala	5'-CCGGTGGGTACCATTGCTACCCCAGAATATCGGATC-3'
P1Pro,P3Pro	5'-CCGGTGGGTACCATTCCAACCCCAGAATATCGGATC-3'
P1Pro,P2Arg	5'-CCGGTGGGTACCATTGTGAGACCAGAATATCGGATC-3'
P1Pro,P2Pro	5'-CCGGTGGGTACCATTGTGCCACCAGAATATCGGATC-3'
P1Pro,P2Glu	5'-CCGGTGGGTACCATTGTGGAACCAGAATATCGGATC-3'
P1Pro,P2Val	5'-CCGGTGGGTACCATTGTGGTTCCAGAATATCGGATC-3'
P1Pro,P2Tyr	5'-CCGGTGGGTACCATTGTGTACCCAGAATATCGGATC-3'
P1Pro,P3Glu	5'-CCGGTGGGTACCATTGAAACCCCAGAATATCGGATC-3'

The sequence of the other primer used for generation of PCR products, and which has homology to the T_{TPI} terminater region is: 5'-TTAAGTGGCTCAGAATG-3'

Expression of mutant CI-2A inhibitors in yeast

Plasmids prepared as described above were transformed into a *S. cerevisiae* strain carrying deletions in the TPI gene by selecting for growth on glucose.

The transformed yeast strains were grown on YPD medium (Sherman, F. et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory 1981). 100 ml medium in shake-flasks was inoculated with individual transformants and shaken at 30°C for approx. 48 hours after which the inhibitor could be purified from the medium.

EXAMPLE 2

Expression of barley chymotrypsin inhibitor CI-2A in *Aspergillus oryzae*

Plasmid constructions

Cloning and expression of *Humicola lanuginosa* lipase in *Aspergillus oryzae* is described in EP 305,216. The same host/vector system can be used for expression and secretion of barley chymotrypsin inhibitor CI-2A. The lipase expression plasmid is termed p960 and makes use of the *A. oryzae* TAKA amylase promoter for driving the transcription and the *Aspergillus niger* glucoamylase transcription terminator.

The plasmid p960 was slightly modified in order to obtain a vector for cloning the inhibitor gene. p960 was digested with NruI and BamHI restriction enzymes. Between these two sites the BamHI/NheI fragment from pBR322, in which the NheI-site was filled in with Klenow polymerase and dNTP's, was cloned, thereby creating plasmid pAO1 (Fig. 2) which contains unique BamHI and NheI sites facilitating cloning of BamHI/XbaI fragments.

A BamHI/AvaI linker with the sequence

```
BamHI
GATCCACCATGAGGAGCTCCCTTGTGCTGTTCTTTGTCTCTGCGTGGACGGCCTTGGCCAGTC
    GTGGTACTCCTCGAGGGAACACGACAAGAAACAGAGACGCACCTGCCGGAACCGGTCAG
        MetArgSerSerLeuValLeuPhePheValSerAlaTrpThrAlaLeuAlaSerP

CTATTCGTCGAAGCTCAGTGGAGAAGAAGC     AvaI
GATAAGCAGCTTCGAGTCACCTCTTCTTCGGGCT
roIleArgArgSerSerValGluLysLysPro
```

encoding the *Humicola lanuginosa* lipase pre-pro sequence and part of the CI-2A inhibitor was combined with the AvaI/XbaI fragment from pYACI2 and cloned into pAO1 digested with BamHI and NheI, thereby creating the expression plasmid pAHLCI2 (Fig. 3).

Transformation of *Aspergillus oryzae* (general procedure)

100 ml of YPD (Sherman et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) was inoculated with spores of *A. oryzae* and incubated with shaking for about 24 hours. The mycelium was harvested by filtration through miracloth and washed with 200 ml of 0.6 M $MgSO_4$. The mycelium was suspended in 15 ml of 1.2 M $MgSO_4$, 10 mM NaH_2PO_4, pH = 5.8. The suspension was cooled on ice and 1 ml of buffer containing 120 mg of Novozym® 234, batch 1687 was added. After 5 min., 1 ml of 12 mg/ml BSA (Sigma type H25) was added and incubation with gentle agitation continued for 1.5 - 2.5 hours at 37°C until a large number of protoplasts was visible in a sample inspected under the microscope.

The suspension was filtered through miracloth, the filtrate transferred to a sterile tube and overlayed with 5 ml of 0.6 M sorbitol, 100 mM Tris-HCl, pH = 7.0. Centrifugation was performed for 15 min. at 1000 g and the protoplasts were collected from the top of the $MgSO_4$ cushion. 2 volumes of STC (1.2 M sorbitol, 10 mM Tris-HCl, pH = 7.5, 10 mM $CaCl_2$) were added to the protoplast suspension and the mixture was centrifugated for 5 min. at 1000 g. The protoplast pellet was resuspended in 3 ml of STC and repelleted. This was repeated. Finally, the protoplasts were resuspended in 0.2 - 1 ml of STC.

100 µl of protoplast suspension was mixed with 5 - 25 µg of p3SR2 (an *A. nidulans* amdS gene carrying plasmid described in Hynes et al., Mol. and Cel. Biol., Vol. 3, No. 8, 1430-1439, Aug. 1983) in 10 µl of STC. The mixture was left at room temperature for 25 min. 0.2 ml of 60% PEG 4000 (BDH 29576), 10 mM $CaCl_2$ and 10 mM Tris-HCl, pH = 7.5 was added and carefully mixed (twice) and finally 0.85 ml of the same solution was added and carefully mixed. The mixture was left at room temperature for 25 min., spun at 2.500 g for 15 min. and the pellet was resuspended in 2 ml of 1.2 M sorbitol. After one more sedimentation the protoplasts were spread on minimal plates (Cove, Biochem. Biophys. Acta 113 (1966) 51-56) containing 1.0 M sucrose, pH = 7.0, 10 mM acetamide as nitrogen source and 20 mM CsCl to inhibit background growth. After incubation for 4 - 7 days at 37°C spores were picked, suspended in sterile water and spread for single colonies. This procedure was repeated and spores of

a single colony after the second reisolation were stored as a defined transformant.

Expression of the barley inhibitor CI-2A in *A. oryzae*

pAHLCI2 was transformed into *A. oryzae* IFO 4177 by cotransformation with p3SR2 containing the amdS gene from *A. nidulans* as described above. Protoplasts prepared as described were incubated with a mixture of equal amounts of pAHLCI2 and p3SR2, approximately 5 μg of each were used. 9 transformants which could use acetamide as sole nitrogen source were reisolated twice. After growth on YPD for three days, culture supernatants were analyzed for inhibitor activity.

EXAMPLE 3

Purification of the wild-type CI-2 inhibitor and mutants thereof

Fermentation broths containing either the wild-type CI-2 inhibitor or one of the following CI-2 inhibitor mutants: CI-2(I56W), CI-2(V57E), CI-2(V57P), CI-2(T58E), CI-2(T58V), CI-2(T58Y), CI-2(T58P), CI-2(M59E), CI-2(M59V), CI-2(M59R), CI-2(M59Y) and CI-2(M59P), produced as described in WO 92/05239, and CI-2(V57E+M59P) produced as described in Example 1, were filtered on a pressure filter (Zeitz K 250-Neu) provided with 0.5% filter aid, and subsequently on a Zeitz EK-1 filter provided with 0.5% filter aid. The filtrate was applied to a Sephadex G25 gelfiltration column equilibrated in 20 mM sodium acetate, pH 4.4. The gelfiltrated protein was adsorbed onto S-Sepharose column material and after washing the column material with 20 mM sodium acetate, pH 4.4, protein was eluted from the material with a 20 mM sodium borate, pH 9.6 buffer (pH 10.2 was used for the basic mutants). The eluate was subjected chromatography on a Q-Sepharose column equilibrated in 20 mM sodium borate, pH 9.6 (10.2). The column was eluted with a linear gradient between 20 mM sodium borate, pH 9.6 (10.2) and the same buffer supplemented with 1M NaCl. Inhibitor-containing fractions were pooled and the buffer was changed to 20 mM sodium acetate, pH 4.4 again using a Sephadex G25 column. The gelfiltrated protein was subjected

to chromtography on a S-Sepharose column equilibrated in 20 mM sodium acetate, pH 4.4. Elution of the column was performed with a linear gradient between the equilibration buffer and the equilibration buffer supplemented with 1M NaCl. Finally, inhibitor-containing fractions were collected and used in the subsequent experiments.

EXAMPLE 4

Interaction of protease with inhibitor containing a single substitution

The interaction of Alcalase® with wild-type CI-2, CI-2(I56W), CI-2(V57E), CI-2(V57P), CI-2(T58E), CI-2(T58V), CI-2(T58Y), CI-2(T58P), CI-2(M59E), CI-2(M59V), CI-2(M59R), CI-2(M59Y) and CI-2(M59P), respectively, was studied in a 0.1 M Tris-HCl buffer, pH 8.6, at 25°C, using the synthetic peptide substrates Suc-Ala-Ala-Pro-Phe-pNA and Suc-Ala-Ala-Ala-pNA (both available from Sigma) to determine residual activity after reacting the protease with the inhibitor in amounts from 0 to 1.5 times the protease concentration.

The following dissociation constants were determined using non-linear regression essentially as described in M. Tashiro et al., Agric. Biol. Chem. 55(1), 1991, pp. 265-267.

Inhibitor	Dissociation constant (K_d)
Wild-type CI-2	8×10^{-14} M
CI-2(I56W) (mutated in P4)	as wild-type CI-2
CI-2(V57E) (mutated in P3)	3×10^{-12} M
CI-2(V57P) (mutated in P3)	3×10^{-10} M
CI-2(T58E) (mutated in P2)	4×10^{-12} M
CI-2(T58V) (mutated in P2)	5×10^{-12} M
CI-2(T58Y) (mutated in P2)	1×10^{-11} M
CI-2(T58P) (mutated in P2)	2×10^{-11} M
CI-2(M59E) (mutated in P1)	4×10^{-12} M
CI-2(M59V) (mutated in P1)	5×10^{-12} M
CI-2(M59R) (mutated in P1)	6×10^{-12} M
CI-2(M59Y) (mutated in P1)	as wild-type CI-2
CI-2(M59P) (mutated in P1)	8×10^{-9} M

The results show that it is possible to change the dissociation constant by several orders of magnitude by single amino acid substitutions in the binding region of the inhibitor.

EXAMPLE 5

Interaction af protease with inhibitor containing two substitutions

The interaction of Alcalase with CI-2(V57E+M59P) was studied in 0.1 M Tris-HCl buffer, pH 8.6, at 25 °C, using the synthetic peptide substrate Suc-Ala-Ala-Ala-pNA to determine residual activity after reacting the protease with the inhibitor in amounts from 0 to 1.5 times the protease concentration. The dissociation constant was determined as described above (Example 4).

Inhibitor	Dissociation constant (K_d)
CI-2(V57E+M59P) (mutated in P3 and P1)	5×10^{-7} M

Comparing this dissociation constant with the dissociation constants for CI-2(V57E) and CI-2(M59P) indicates that the P3 and P1 sites act independently.

EXAMPLE 6

The protection of lipase from proteolytic degradation in the presence of a protease inhibitor was determined by preparing aqueous solutions of 78 μM *Humicola lanuginosa* lipase (Lipolase® available from Novo Nordisk A/S) with or without 1.9 μM protease and with or without protease inhibitor (1.9 μM or 5.6 μM) in 50 mM Tris-HCl, pH 8.0. The protease used was Savinase® (available from Novo Nordisk A/S) and the protease inhibitor used was CI-2(M59P). The solutions were stored at room temperature for up to 20 days. Lipase activity was measured before and after storage and expressed as % residual activity.

The results are shown in Figure 4. It appears that the lipase (L) alone is very stable, but it is strongly destabilized by the protease (S). It further appears that the protease inhibitor (I) has a stabilizing effect on the lipase in the presence of protease.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Novo Nordisk A/S

(ii) TITLE OF INVENTION: Detergent containing protease and inhibitor and novel inhibitor

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Novo Nordisk A/S
(B) STREET: Novo Alle
(C) CITY: Bagsvaerd
(E) COUNTRY: Denmark
(F) ZIP: 2880

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Thalsoe-Madsen, Birgit
(C) REFERENCE/DOCKET NUMBER: 3749.000-DK

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: +4544448888
(B) TELEFAX: +4544493256
(C) TELEX: 37304

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 592 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: barley

(ix) FEATURE:
(A) NAME/KEY: CDS

(B) LOCATION: 77..580

(ix) FEATURE:
(A) NAME/KEY: sig_peptide
(B) LOCATION: 77..331

(ix) FEATURE:
(A) NAME/KEY: mat_peptide
(B) LOCATION: 332..580

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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GAT GAA ACG GCA CAA ATT CCG GCT GAA GCT GTC ATC GGT TAC TCA GAT        205
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ACC ATT GTG ACC ATG GAA TAT CGG ATC GAT CGC GTC CGC CTC TTT GTC        541
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75 80

GA 592

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 168 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Arg Phe Pro Ser Ile Phe Thr Ala Val Leu Phe Ala Ala Ser Ser
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-65 -60 -55

Ile Pro Ala Glu Ala Val Ile Gly Tyr Ser Asp Leu Glu Gly Asp Phe
-50 -45 -40

Asp Val Ala Val Leu Pro Phe Ser Asn Ser Thr Asn Asn Gly Leu Leu
-35 -30 -25

Phe Ile Asn Thr Thr Ile Ala Ser Ile Ala Ala Lys Glu Glu Gly Val
-20 -15 -10

Ser Leu Asp Lys Arg Ser Ser Val Glu Lys Lys Pro Glu Gly Val Asn
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Thr Gly Ala Gly Asp Arg His Asn Leu Lys Thr Glu Trp Pro Glu Leu
15 20 25

Val Gly Lys Ser Val Glu Glu Ala Lys Lys Val Ile Leu Gln Asp Lys
30 35 40

Pro Glu Ala Gln Ile Ile Val Leu Pro Val Gly Thr Ile Val Thr Met
45 50 55

Glu Tyr Arg Ile Asp Arg Val Arg Leu Phe Val Asp Lys Leu Asp Asn
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Ile Ala Gln Val Pro Arg Val Gly
80

CLAIMS

1. A detergent composition comprising a protease and a modified subtilisin inhibitor of Family VI having Pro as the P1 residue in combination with one or more of the following amino acid substitutions at the indicated positions:

P4: Pro
P3: Tyr, Glu, Ala, Arg or Pro
P2: Arg, Pro, Glu, Val or Tyr.

2. A detergent composition according to claim 1, characterized by an dissociation constant in the range from 0.05 to 50 μM.

3. A composition according to Claim 2, wherein said constant is in the range from 1 to 10 μM.

4. A composition according to any of Claims 1 - 3, wherein the protease is a serine protease, preferably an alkaline microbial protease or a trypsin-like protease.

5. A composition according to Claim 4, wherein the trypsin-like protease is trypsin or is derived from *Fusarium*.

6. A composition according to Claim 4, wherein the alkaline microbial protease is a subtilisin.

7. A composition according to Claim 1, wherein the inhibitor is a modified barley subtilisin inhibitor CI-1 or CI-2, potato subtilisin inhibitor (PSI), Eglin B or C, tomato subtilisin inhibitor or *Vicia* subtilisin inhibitor (VSI).

8. A composition according to Claim 6, wherein the subtilisin is derived from *Bacillus* and is preferably subtilisin Novo, subtilisin Carlsberg, BPN', subtilisin 309, subtilisin 147 or subtilisin 168.

9. A composition according to any of the preceding claims, wherein the amount of protease is 0.2-40 μM, preferably 1-20 μM.

10. A composition according to any of the preceding claims, additionally comprising another enzyme, in particular an amylase, lipase, cellulase or peroxidase.

11. A composition according to any of the preceding claims, which is in the form of an aqueous liquid.

12. A detergent composition according to any of claims 1-11, wherein the degree of protease inhibition in the detergent is at least 60%, and the degree of protease inhibition in a 1% detergent solution in water is below 10%.

13. A detergent additive comprising a protease in the form of a stabilized liquid or a non-dusting granulate, further comprising a modified subtilisin inhibitor of Family VI having Pro as the P1 residue in combination with one or more of the following amino acid substitutions at the indicated positions:

P4: Pro
P3: Tyr, Glu, Ala, Arg or Pro
P2: Arg, Pro, Glu, Val or Tyr.

14. A detergent additive according to claim 13, which additionally comprises another enzyme, in particular an amylase, lipase, cellulase or peroxidase.

15. A modified subtilisin inhibitor of Family VI having Pro as the P1 residue in combination with one or more of the following amino acid substitutions at the indicated positions:

P4: Pro,

P3: Tyr, Glu, Ala, Arg or Pro,

P2: Arg, Pro, Glu, Val or Tyr,

excluding:

Eglin B and C substituted with

Pro at position 44 (P2),

16. A modified subtilisin inhibitor according to claim 15, which is a modified barley subtilisin inhibitor CI-1 or CI-2, potato subtilisin inhibitor (PSI), Eglin B or C, tomato subtilisin inhibitor or *Vicia* subtilisin inhibitor (VSI).

17. A recombinant DNA molecule comprising a nucleotide sequence coding for the modified subtilisin inhibitor of Claim 15 or 16.

18. A transformed host organism comprising the DNA of Claim 17.

19. A method of producing the inhibitor of Claim 15 or 16, comprising culturing the transformed host organism of Claim 18 in a suitable culture medium under conditions permitting the expression of the nucleotide sequence coding for the modified inhibitor, and recovering the inhibitor from the culture.

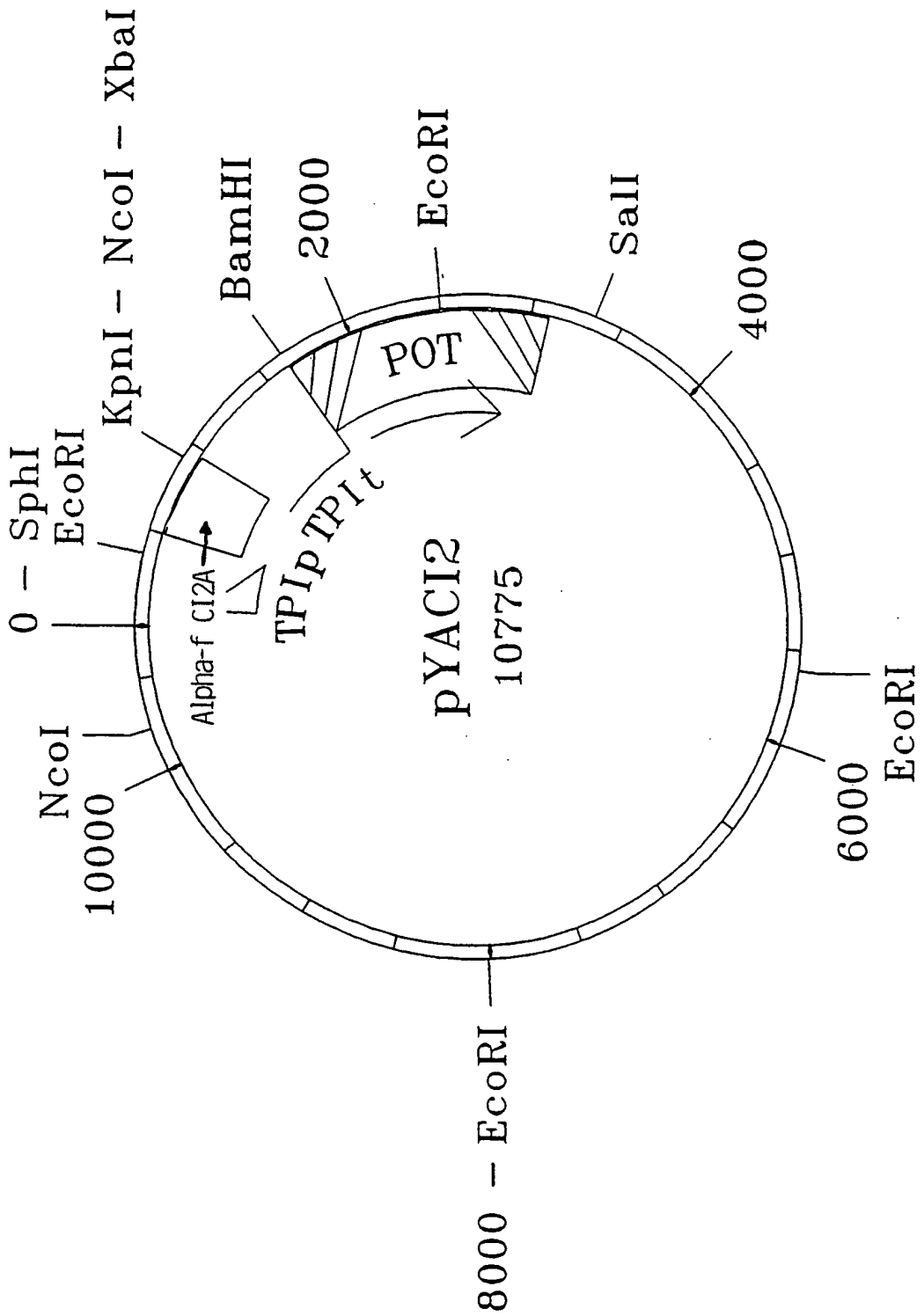
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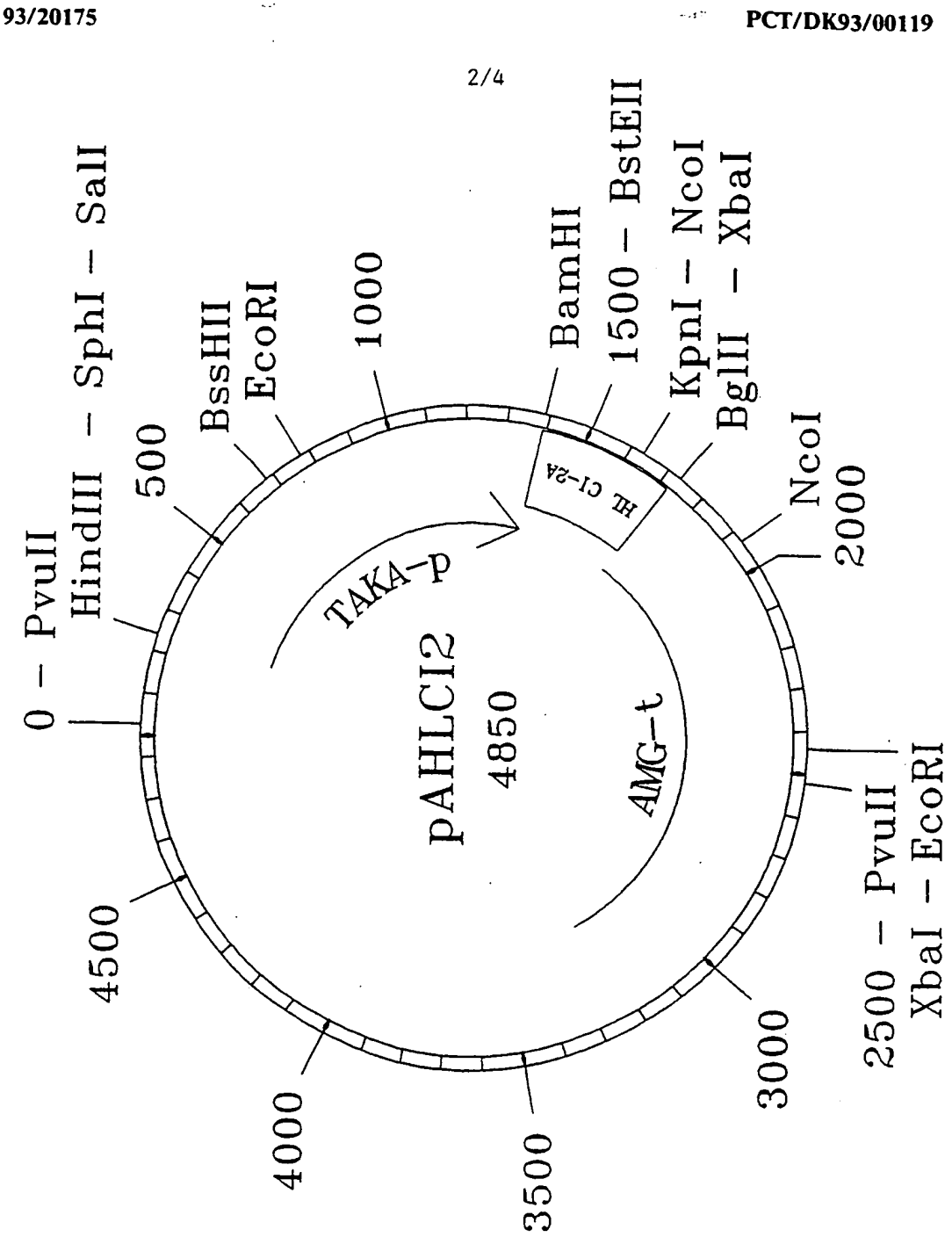
Fig. 1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BamHI
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NcoI
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2500 – PvuII
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Fig. 2

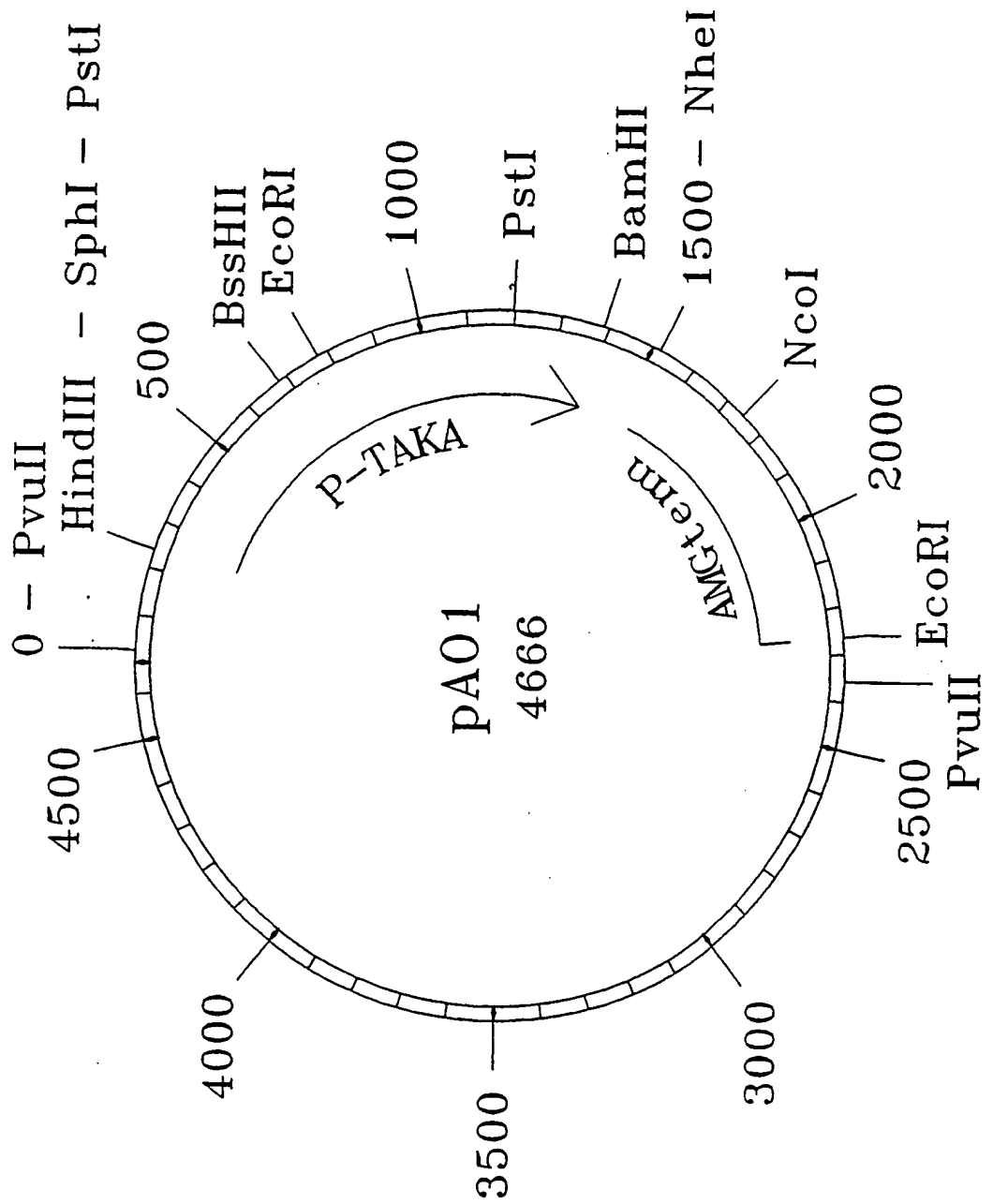
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Fig. 3

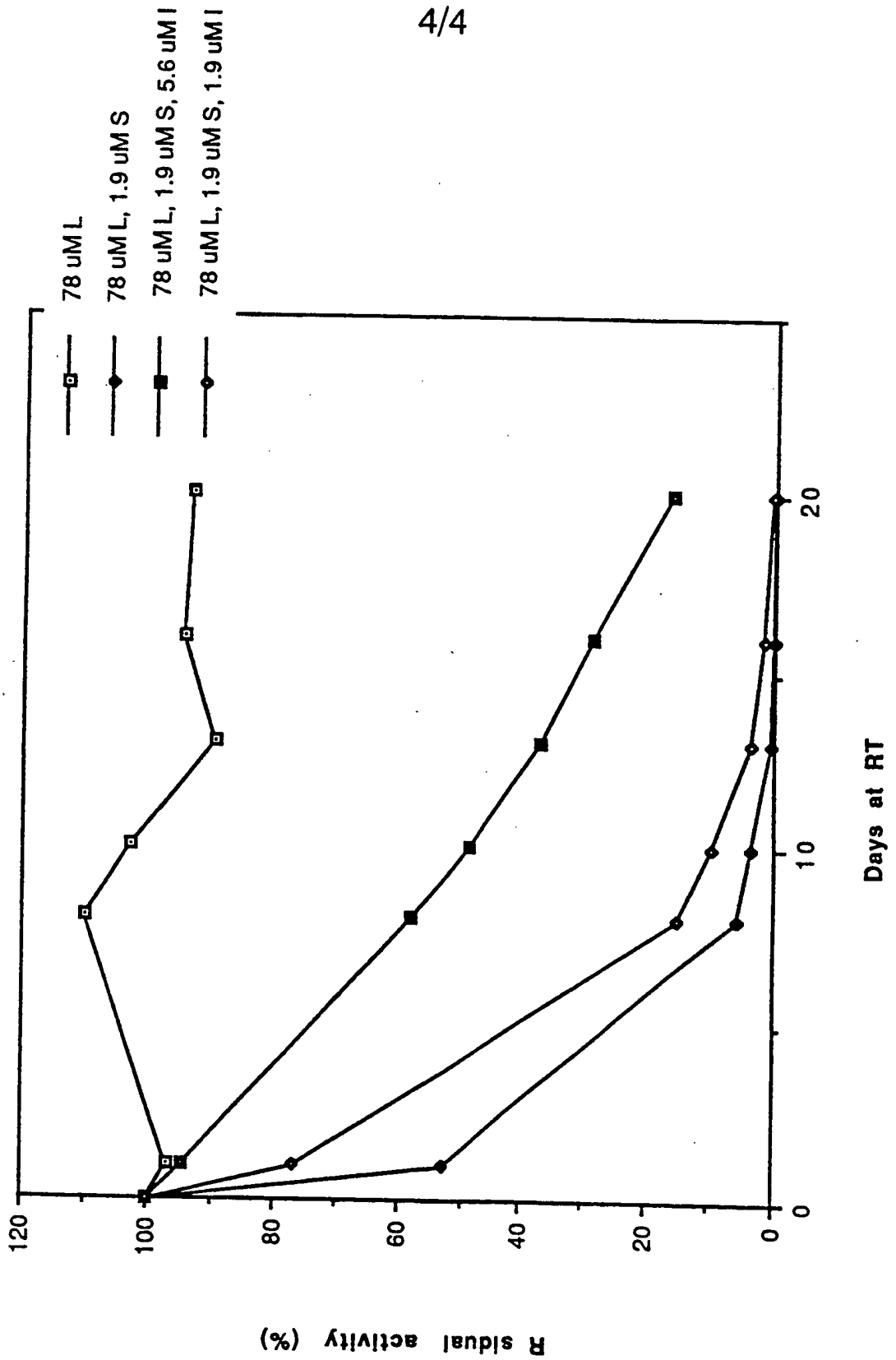
78 uM L
78 uM L, 1.9 uM S
78 uM L, 1.9 uM S, 5.6 uM I
78 uM L, 1.9 uM S, 1.9 uM I
R sidual activity (%)
120
100
80
60
40
20
0
0
10
20
Days at RT

Fig. 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 93/00119

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: C11D 3/386
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: C11D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO, A1, 9205239 (NOVO NORDISK A/S), 2 April 1992 (02.04.92)	1-19
X	WO, A1, 9203529 (NOVO NORDISK A/S), 5 March 1992 (05.03.92)	1-13
A	US, A, 5039446 (ESTELL), 13 August 1991 (13.08.91)	1-12

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"B" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 July 1993	08 -07- 1993
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Dagmar Järvman Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT
Information on patent family members

28/05/93

Intern... application No.
PCT/DK 93/00119

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9205239	02/04/92	NONE	
WO-A1- 9203529	05/03/92	NONE	
US-A- 5039446	13/08/91	NONE	